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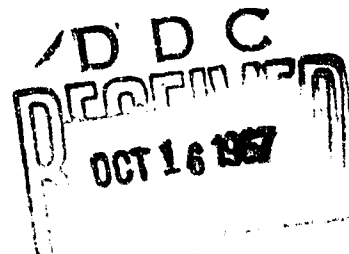
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TECHNICAL MANUSCRIPT 404

ANTHRAX TOXIN:
PRIMARY SITE OF ACTION

Norman S. Remmele
Frederick Klein
James A. Vick
Jerry S. Walker
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JULY 1967



DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

The site of action of Bacillus anthracis toxin in the rhesus monkey was the central nervous system (CNS). Injection of toxin directly into the cerebrospinal fluid produced death in 6 to 10 minutes, in contrast with the 30 hours required to kill when 10 times this dose was given intravenously. During this short period of toxemia, the monitored physiological systems changed drastically, indicative of a tremendous increase in CNS discharges, resulting in terminal anoxia. Inactivation of the toxin with antiserum or heat prevented the lethal effect; the animals survived. If respiration could be maintained, the animals also survived toxin challenge. Maintenance of respiration was achieved by artificial ventilation and reestablished by the intravenous injection of a beta adrenergic stimulant, isoproterenol hydrochloride (Isuprel).

I. INTRODUCTION

Since Keppie et al.¹ demonstrated that a lethal toxin was liberated during in vivo growth of Bacillus anthracis, site of action and mechanism of death have been the subject of study and some controversy. The principal theories advanced by workers since 1955 of mechanism of death are: (i) secondary shock, defined² as a pathological state involving circulatory failure; (ii) asphyxia, because extremely low blood O₂ was noted by Nordberg et al.³ as the actual cause of death by anthrax; and (iii) direct effect on the central nervous system (CNS) as the primary site of action.⁴ This last hypothesis was verified by Klein et al.,⁵ who showed depression of the electroencephalogram by experimental anthrax. In addition, working only with the toxin per se, Eckert and Bonventre⁶ verified that asphyxia occurred in rats following lethal challenge. The principal action of this phenomenon in the rat was interpreted by Beall and Dalldorf⁷ as increased permeability of the pulmonary vasculature and by Gray and Archer⁸ as inhibition in lung tissue of regeneration of nicotinamide adenine dinucleotide. Vick et al.⁹ showed depression of the central cortical electrical activity and, more specifically, the central respiratory mechanism of monkeys challenged with toxin. Their data show no cardiac changes until immediately before death. The study by Klein et al.¹⁰ of the pathophysiology of monkeys, chimpanzees, and rabbits dying of anthrax or of toxin challenge confirms the terminal hypoxia and a remarkable parallel response between spore- and toxin-challenged hosts; however, they fail to demonstrate a kidney shutdown that accompanies "secondary shock."

With this background of conflicting hypotheses of death by anthrax, we considered it of interest to define more specifically the primary site of action of anthrax toxin. This study, therefore, was concerned with the physiological responses obtained when the toxin was placed in direct contact with the proposed CNS site and with the possibility of preventing death by supporting the affected physiological system.

II. MATERIALS AND METHODS

Anthrax toxin was prepared and its potency was determined by a method previously described by Haines et al.,¹¹ with the exception that horse serum was not added after centrifugation and the toxin was lyophilized after filtration. The toxin was then stored at -20 C. Each tube of toxin was reconstituted with sterile saline to the desired concentration. The chemical characteristics of the toxin are described elsewhere.^{9,12}

Rhesus monkeys (Macaca mulatta) weighing 3.0 to 3.5 kg were used for this experiment. Prior to the injection of toxin, each animal was anesthetized with 30 mg pentobarbital sodium per kg of body weight. The toxin was injected directly into the cerebrospinal fluid via the cisterna magna. After subdural placement of the needle, 1 ml of cerebrospinal fluid was removed and the 1.5 ml of toxin containing 1,000 rat units were injected.

Arterial and venous blood pressure were monitored continuously with No. 54 polyethylene tubing connected to a Stantham strain gauge and recorded on an E & M physiograph. Heart rate, electrocardiogram (EKG), and respiratory rate were also recorded on the physiograph, using a pair of needle-tipped electrodes placed subcutaneously into either side of the chest wall. The heart rate was recorded so that as heart rate increased, the width of the trace decreased (Fig. 1, 3, 4, and 5), and vice versa. Animals living after 72 hours postchallenge were considered permanent survivors.

III. RESULTS

A. PHYSIOLOGICAL EFFECTS OF THE TOXIN

When 1,000 rat units of anthrax toxin were injected into the cerebrospinal fluid, the monkeys expired at 6 to 10 minutes, which contrasts with the 30 hours required to kill when 10,000 rat units were administered intravenously (IV).⁹

The pattern of death could be divided into three distinct phases on the basis of clinical observation and monitored physiological responses (Fig. 1). The first phase was characterized clinically by tetanic paralysis and complete cessation of respiration, which followed the injection of toxin by a few seconds. Marked changes occurred physiologically within 15 to 20 seconds postinjection. The tetanic paralysis and severe muscle contractions produced artifactual changes superimposed on the respiratory trace. Thus

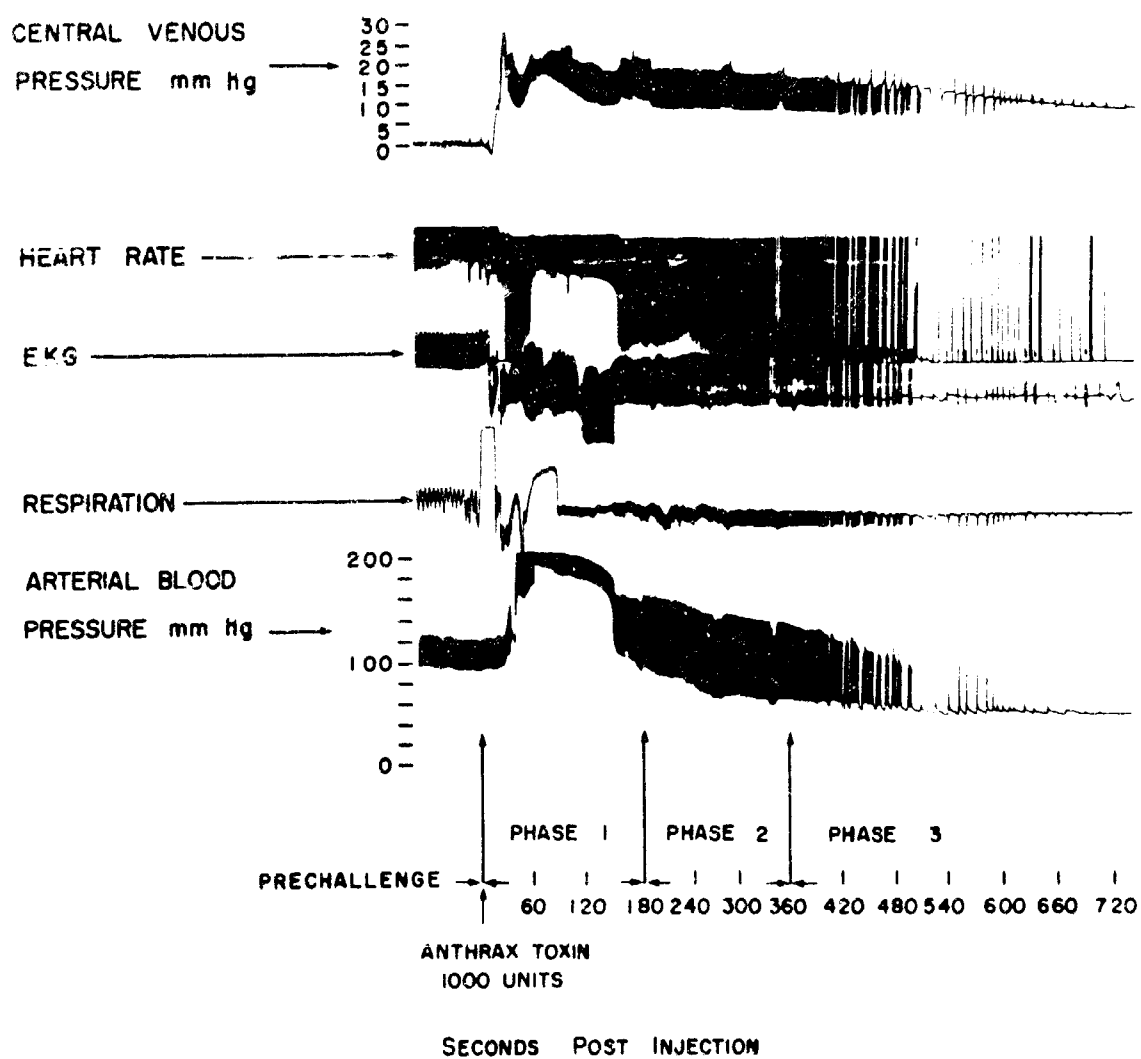


Figure 1. Physiological Responses in the Rhesus Monkey Following Challenge of 1,000 Units Anthrax Toxin into Cerebrospinal Fluid.

the respiratory patterns recorded soon after injection of toxin are related to violent and severe muscle contraction and not to respiration per se. Also, during the first 15 seconds, central venous pressure increased sharply from a control of 1 to 2 mm of Hg to a maximum of 20 to 24 mm of Hg; at the same time, arterial pressure rose from 120 to 200 mm of Hg with some decrease in pulse pressure. Simultaneously the heart rate decreased from an average of 130 beats per minute; however, this was quickly followed by a return to normal. At approximately 180 seconds postinjection, changes in EKG were observed, although they are not apparent in Figure 1 because of recording techniques. These changes appear consistent with those in myocardial hypoxia (prolongation of the P-R interval, depression of the T wave, and inversion of the QRS segment).

In the second phase, from 180 to 360 seconds, intensive muscle fasciculation developed that was extremely noticeable clinically as well as physiologically. This fasciculation was reflected in the central venous and arterial blood pressure traces. The arterial blood pressure began to decrease, with some slowing of the heart rate. The EKG remained abnormal, as in the first phase, and some feeble attempts at respiration were made.

The terminal phase, from 360 seconds to death, was characterized clinically by a loss of muscle rigidity and by a generalized flaccidity. Prolonged and progressive hypotension was evident by 360 seconds when the diastolic pressure reached levels of about 25 mm of Hg. The pulse pressure was markedly increased and central venous pressure remained above normal until death. The heart rate became irregular and progressively slower, terminating in complete arrest. An increased and inverted T wave was evident from the EKG tracings, again typical of myocardial hypoxia. Death at 420 to 600 seconds followed respiratory embarrassment that began within seconds postinjection and continued throughout all three phases.

A surgically implanted aortic flow probe and a monitoring of the right and left heart blood pressures revealed the following changes: (i) within 15 seconds after injection of the toxin into the cerebrospinal fluid, the aortic flow dropped from 780 to 290 ml per minute and at 150 seconds the flow was recorded as 0 (Fig. 2); (ii) the left heart pressure dropped parallel to that of the aorta; (iii) the right heart pressure rose sharply as shown by the rise in central venous pressure (Fig. 1).

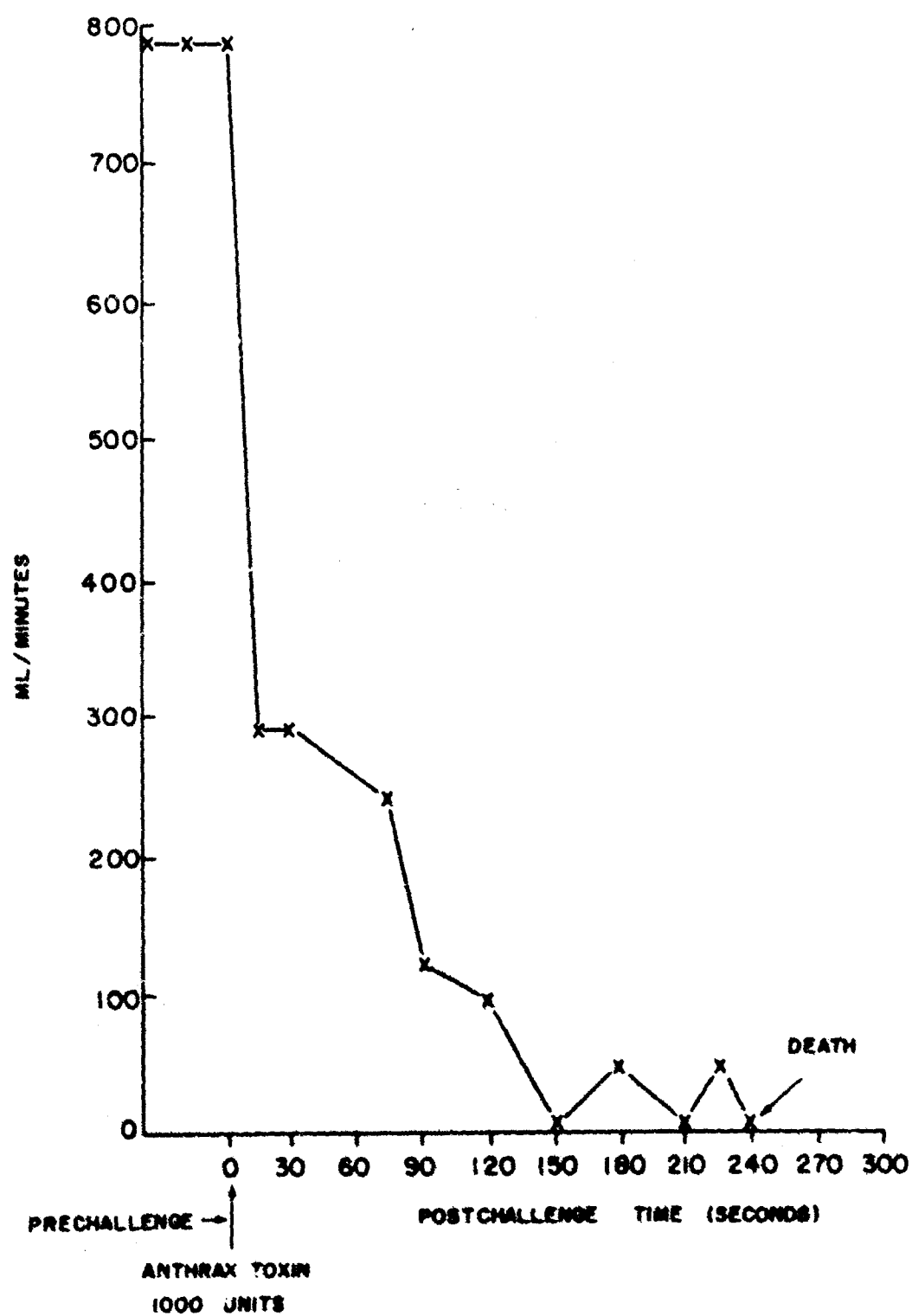


Figure 1. Flow Rate in Aorta Following Challenge of 1,000 Units of Anthrax Toxin.

E. CONTROLS

To differentiate the action of the toxin from nonspecific responses, several controls were included. For volume and diluent controls, three monkeys were given subdural injections of 1, 3, and 5 ml of sterile saline. In addition, 3 ml of cerebrospinal fluid were injected into a fourth monkey as a volume control. For toxin controls, inactivated toxin was prepared by two methods and injected into several monkeys. By the first method, 1,000 rat units of toxin were reconstituted with equine antiserum (7,000 neutralizing units per ml) in place of the normal saline diluent. This antiserum-neutralized toxin did not kill either rats or monkeys. By the second method, 1,000 rat units of toxin in saline were boiled in a water bath for 60 minutes, a treatment that has previously been shown⁹ to inactivate the lethal activity of the toxin without affecting serological activity.¹²

No abnormal physiological or clinical responses were noted with any of the volume or diluent controls. After injection of the inactivated toxin (both heat- and antiserum-inactivated), the physiological and clinical parameters were similar to those of the regular toxin challenge for the first two phases except that respiration continued very erratically (Fig. 3). However, after 6 to 8 minutes, respiration had returned to normal and, although more regular, it was somewhat slower and deeper. The heart rate and EKG also returned toward normal at the same time, as evidenced by amplitude of the heart rate recording. The continuation of an inverted T wave indicated that myocardial hypoxia still existed. Upon recovery from anesthesia, the animals appeared clinically normal and all four (two with antiserum- and two with heat-inactivated toxin) survived.

C. TREATMENTS TO PREVENT LETHAL EFFECTS OF TOXIN

From the data presented here and conclusions drawn from other papers,^{5,9} it appeared that if respiration could be maintained or the smooth muscle contractions overcome (as exemplified by vasoconstriction), the lethal effects of the toxin could be prevented.

1. Artificial Ventilation

To test the effect of maintaining respiration, animals were placed on a positive-pressure respirator prior to administration of the toxin via the cerebrospinal fluid and maintained in this manner for 10 minutes post-injection. Figure 4 reproduces tracings before and after challenge that are typical of the five animals tested. Again, within seconds after toxin challenge, the blood pressure increased. It is important to note, however, that the increase in arterial blood pressure was such that the upper limits of the recording apparatus (galvanometer) were exceeded. The EKG became

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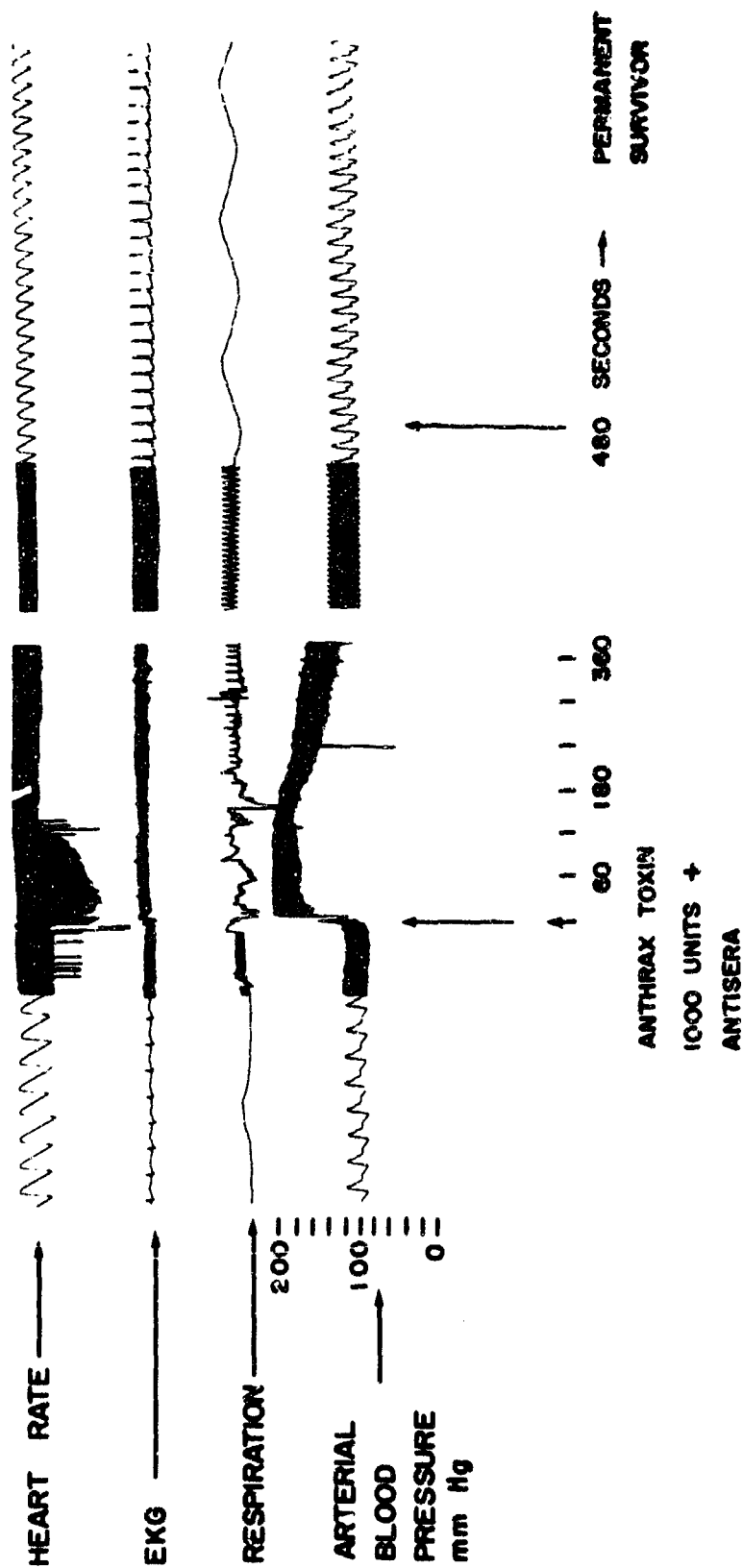
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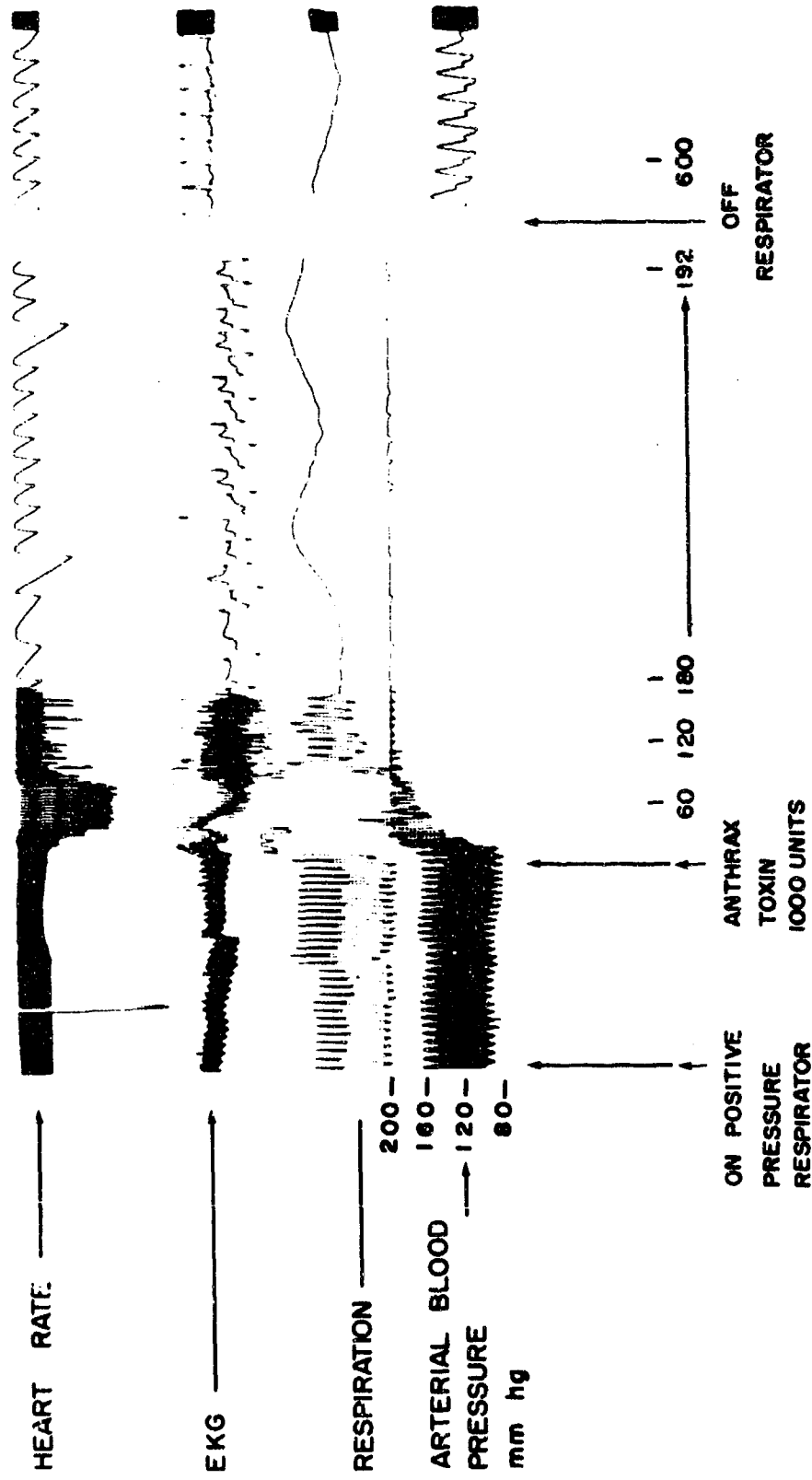
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SECONDS POST INJECTION

Figure 3. Response of Rhesus Monkey Following Challenge of 1,000 Units of Anthrax Toxin + Equine Antiserum into Cerebrospinal Fluid.



SECONDS POST INJECTION

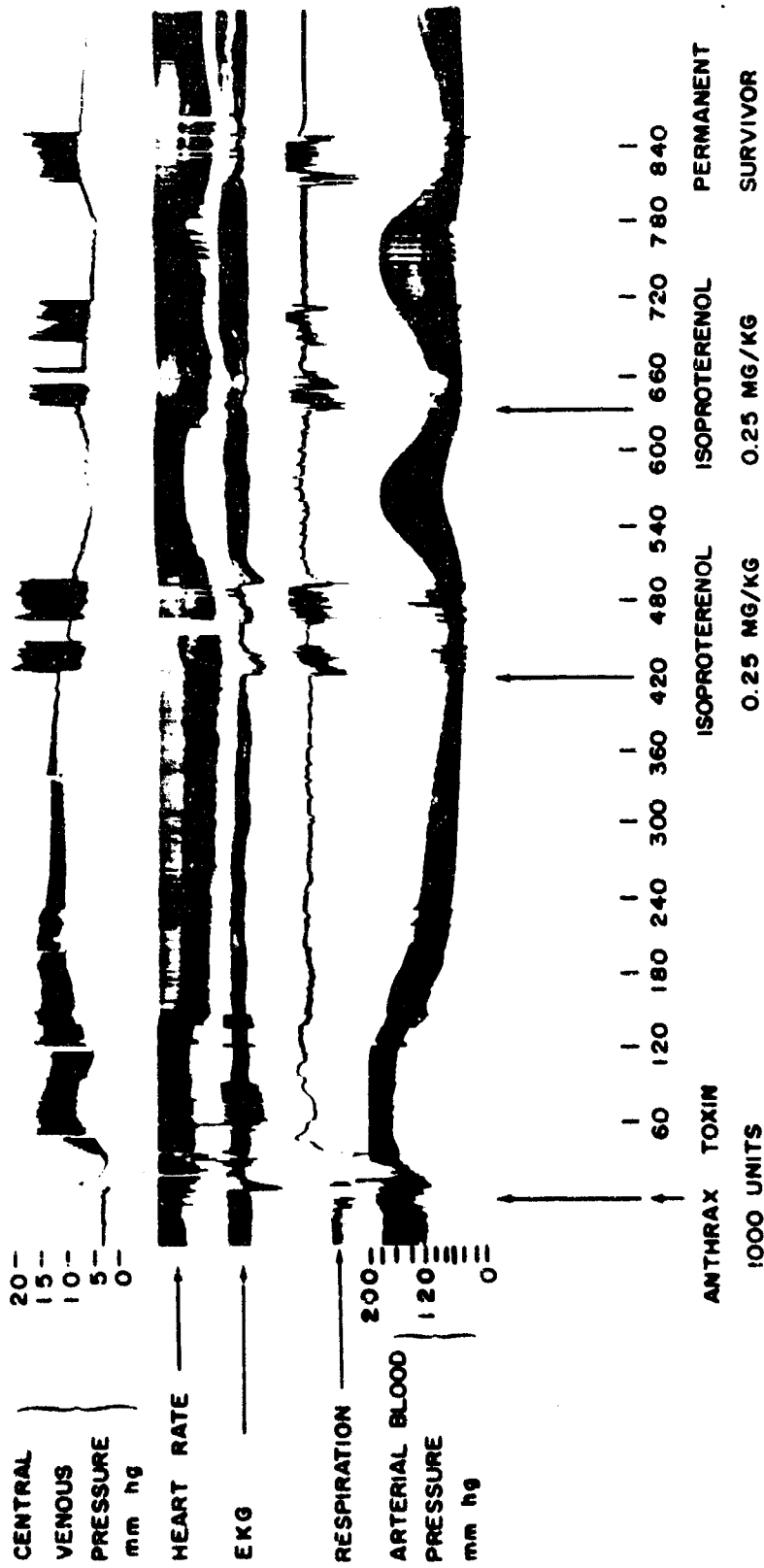
Figure 4. Artificial Respiratory Maintenance Following Subdural Injection of Anthrax Toxin.

abnormal and the heart rate slower. The tracings for respiration showed abnormalities that resulted from the tremendous muscle spasms that accompanied tetanic paralysis and general muscle contraction. Clinically, however, the respiration continued at regular intervals because the animals were on the positive-pressure respirator. Adequate ventilation was provided to the animals at all times. On the expanded tracings in Figure 4 (180 to 192 seconds), irregularities in heart rate and EKG are apparent. These changes were for the most part due to the A-V disassociation and undoubtedly again to myocardial hypoxia. The blood pressure remained high and did not fall at about 180 seconds or during phase two without maintenance of respiration, as previously noted in Figure 1. At 10 minutes the animals were taken off the respirator and were able to maintain their own respiration as evidenced by the tracings in Figure 4 and visual observation. Blood pressure and heart rate had returned to normal. The EKG still showed some effects of myocardial hypoxia but was greatly improved over that observed at 180 seconds. All five animals recovered without any apparent ill effects.

2. Treatment with Isoproterenol

The second approach to overcome the lethal effects of toxin was the use of a beta adrenergic stimulant, dl-isoproterenol hydrochloride.* Figure 5 shows the tracing obtained from a monkey given 1,000 rat units of toxin via the cerebrospinal fluid and then treated with isoproterenol. The clinical and physiological effects before administration of isoproterenol were similar, if not identical, to those shown in Figure 1. At 420 seconds, when the blood pressure had reached severe hypotension, i.e., diastolic pressure less than 25 mm of Hg, 0.25 mg isoproterenol per kg of body weight was administered slowly IV. Blood pressure increased so that at approximately 540 seconds it was above normal and the central venous pressure had returned toward normal. Heart rate increased. The response of the respiratory system was dramatic in that periods of hyperventilation followed the IV infusion of isoproterenol. Following hyperventilation, periods of irregular respiration were noted (Fig. 5). The effect of isoproterenol in respiration is reflected in both the arterial and central venous tracings. These changes are viewed as degree increases in the magnitude of trace and reflect increased intrathoracic pressure. However, by 630 seconds the blood pressure started to fall and a second infusion of isoproterenol administered at the same dose level resulted in responses identical to that following the first injection. After 14 minutes, the monitored systems tended to become normal. The respiration had become more regular as observed clinically, and all five animals treated with isoproterenol survived.

* Isuprel, Winthrop Laboratories, 90 Park Ave., New York, New York.



SECONDS POST INJECTION

Figure 5. Isuprel Used as Supportive Therapy Following Subdural Injection of Anthrax Toxin.

IV. DISCUSSION

Considering the low dosage of toxin required to kill in about 10 minutes and the drastically altered physiology of the host, there is little doubt that anthrax toxin affects the CNS. The many changes produced following subdural administration of toxin may be explained by a tremendous increase in CNS discharges, which results in severe generalized muscle contraction of both smooth and skeletal muscles, causing first an increase in blood pressure, then a rise in central venous pressure followed by a drop in aortic blood flow. The initial abnormalities in EKG may be accounted for by the same explanation. Changes noted in EKG are typical of myocardial hypoxia, which follows the dyspnea noted within 15 seconds postinjection.

The ultimate effect of the toxin on the CNS is anoxia. This is caused by a lack of oxygenation of the blood either by itself or in combination with decreased blood flow. The terminal anoxia could arise through: (i) a direct effect on the respiratory center in the CNS, causing a respiratory failure and a lack of oxygenation, (ii) a tetanic paralysis of the intercostal and diaphragmatic muscles, arising from increased CNS discharges, again resulting in respiratory paralysis and lack of oxygenation, and (iii) a cardiovascular failure mediated by the increased CNS discharges producing a generalized smooth muscle constriction. The generalized smooth muscle constriction would also cause lack of oxygenation through bronchial constriction.

The results obtained with the inactivated forms of the toxin (inactivated with antiserum or heat) suggest that survival depends upon continuation of respiration, because the toxin component affecting the respiratory center has been inactivated or the components that cause the CNS cardiovascular failure have been altered in such a way that the animal is able to reestablish enough blood flow to prevent terminal anoxia.

Survival obtained following use of isoproterenol may be attributed to dilatation of the pulmonary vasculature, which would allow uninterrupted flow of blood through the lungs, or to the known action of isoproterenol on the myocardium, which causes an increased cardiac output.¹³ Either one or both of these effects could maintain or reestablish the function of the CNS or especially the respiratory center by increasing circulation and oxygenation. The survival obtained by the use of a positive-pressure respirator, however, may be due to one or any combination of the three previously mentioned causes of terminal anoxia.

It is a remote possibility that the dramatic changes produced by subdural administration of the toxin are caused by a component of the toxin that cannot cross the blood-brain barrier in the infection or following IV challenge; however, we do not feel this to be the case. It is more probable that subdural administration accentuates the CNS effects.

Except for cases where specific antiserum was administered, we believe this to be the first demonstration in animals given lethal amounts of bacterial toxin that death can be prevented by sustaining and maintaining the affected primary physiological system. Whatever the ultimate cause of the CNS-mediated anoxia, the therapeutic and supportive treatment of acute bacterial diseases has now taken on a new and exciting aspect.

V. SUMMARY

Administration of anthrax toxin via the cerebrospinal fluid demonstrates positively that it alters the physiology of the body, primarily via the CNS, and kills by terminal anoxia mediated by the CNS. A positive-pressure respirator or isoproterenol prevented death in animals that had received lethal doses of toxin. This demonstration of therapy for effects of a lethal bacterial toxin opens new avenues of investigation.

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